Review

Ca\(^{2+}\)-Translocating ATPase of the Plant Plasma Membrane

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ABSTRACT

For Ca\(^{2+}\) to function as a second messenger in signal transduction, it is essential that plant cells maintain low cytoplasmic Ca\(^{2+}\) levels relative to internal organelles and the apoplast. At the plasma membrane, Ca\(^{2+}\) is actively transported out of the cytoplasm and current evidence supports the involvement of a primary Ca\(^{2+}\)-translocating ATPase in mediating this energy-dependent process. This review examines the preliminary biochemical characterization of this transport enzyme.

The ability of plant cells to maintain a low cytoplasmic concentration of Ca\(^{2+}\) is a key requirement for the function of this divalent cation as a second messenger in the regulation of metabolic events (17 and references therein). Since fluctuations in cytoplasmic Ca\(^{2+}\) concentration may represent an important link in signal transduction, maintenance of low cytoplasmic Ca\(^{2+}\) serves to poise this regulatory system for cellular responses to hormonal or environmental cues. In plant cells, the cytoplasmic Ca\(^{2+}\) concentration is maintained at levels approaching 10\(^{-7}\) M through the action of Ca\(^{2+}\)-transport systems involved in either sequestering this cation into internal organelles (mitochondria, ER, vacuole) or mediating its efflux to the cell exterior (17). With organellar and extracellular Ca\(^{2+}\) levels approaching the millimolar concentration range, this process must occur continuously to transport Ca\(^{2+}\) out of the cytoplasm.

Preparations of isolated membrane vesicles have proven useful for studying Ca\(^{2+}\) transport mechanisms relevant to the maintenance of low cytoplasmic Ca\(^{2+}\) (4 and references therein). Using this approach, it has been possible to demonstrate the presence of transport systems responsible for sequestering Ca\(^{2+}\) into internal organelles such as an H\(^{+}\)/Ca\(^{2+}\) antiport associated with the tonoplast (1), and a Ca\(^{2+}\)-translocating ATPase associated with the ER (11 and references therein). In more recent studies, it has been possible to demonstrate the activity of a Ca\(^{2+}\)-translocating ATPase associated with isolated plasma membrane vesicles (12, 13, 15, 18, 19, 23). This review will focus on this transport system proposed to mediate Ca\(^{2+}\) efflux from the plant cell and will outline recent work to elucidate its biochemical characteristics.

EARLY DIFFICULTIES IN THE DEMONSTRATION OF A UNIQUE Ca\(^{2+}\)-TRANSLOCATING ATPase ASSOCIATED WITH THE PLASMA MEMBRANE

There was initial uncertainty as to whether a primary Ca\(^{2+}\)-translocating ATPase was associated with the plant plasma membrane and involved in mediating Ca\(^{2+}\) efflux from the cytoplasm to the cell exterior. Although early reports in the literature often described an ATP hydrolytic activity associated with plasma membrane fractions (for example, barley roots, [6]) that was stimulated by Ca\(^{2+}\) alone or Ca\(^{2+}\) and Mg\(^{2+}\), these studies were not correlated with any measurements of Ca\(^{2+}\) transport. The relevance of this enzyme activity to Ca\(^{2+}\) transport was further questioned by the observation that these membrane fractions contained substantial levels of a Ca\(^{2+}\)-dependent phosphatase capable of hydrolyzing ATP (10). While an ATP-driven Ca\(^{2+}\) transport activity of putative plasma membrane origin had been examined in isolated vesicles and the ATPase thought to be responsible for this transport purified (9 and references therein), this work was based on the use of a microsomal membrane preparation where it was difficult to ascribe the transport activity to one specific membrane component or another. Further uncertainty was raised with the subsequent demonstration of a Ca\(^{2+}\)-translocating ATPase in ER vesicles (11 and references therein) and the observation that membranes from internal organelles (e.g. ER, vacuole) tended more readily to form transport-competent vesicles while plasma membrane vesicles tended to be 'leaky' and less apt to be active in transport assays (4 and references therein).

The demonstration of a primary ATP-driven Ca\(^{2+}\) transport system in association with the plasma membrane, and hence a unique plasma membrane Ca\(^{2+}\)-translocating ATPase, was eventually achieved with the development of methods for the production of transport-competent plasma membrane vesicles (4 and references therein). In transport-competent plasma membrane vesicles isolated from radish seedlings (18, 19, 20), red beet storage tissue (12), spinach leaves (15), and Commelina communis leaves (13), it has been possible to demonstrate ATP-dependent 45Ca\(^{2+}\) uptake that was insensitive to ionophores which collapse \(\Delta \mu \text{H}^+\). This would suggest direct coupling of Ca\(^{2+}\) transport to ATP hydrolysis rather than secondary transport and the orientation of transport with respect to vesicle sidedness (i.e. ATP can only energize inside-out plasma membrane vesicles) would indicate a transport system involved in mediating efflux from the cell. Taken together, these results were consistent with a primary Ca\(^{2+}\)-translocating ATPase (subsequently referred to as Ca\(^{2+}\)-ATPase) which mediates Ca\(^{2+}\) transport from the cytoplasm to the cell exterior.

Abbreviations: \(\Delta \mu \text{H}^+\), proton electrochemical gradient; CCCP, carbonylcyanide m-chlorophenylhydrazone.
BIOCHEMICAL CHARACTERIZATION OF THE PLASMA MEMBRANE Ca\textsuperscript{2+}-ATPase

Using plasma membrane vesicles, the biochemical characteristics of the Ca\textsuperscript{2+}-ATPase have been determined where most results have been based upon measurements of ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} transport. In kinetic studies, ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} uptake shows simple saturation kinetics when measured as a function of either ATP or Ca\textsuperscript{2+} concentration. While a \( K_m \) of about 0.3 mM was observed if both ATP and Mg\textsuperscript{2+} were varied as the 1:1 concentration ratio (12, 13), this value decreased to about 15 to 20 \( \mu \)M if assays were conducted in the presence of excess Mg\textsuperscript{2+} and only ATP was varied (19). When analyzed in terms of the total Ca\textsuperscript{2+} added to the assay, a \( K_m \) of about 4 to 6 \( \mu \)M was observed (12, 13), and this value decreased to about 70 nM if only the level of free Ca\textsuperscript{2+} was considered (19). These low \( K_m \) values for Ca\textsuperscript{2+} are consistent with a transport enzyme involved in maintaining cytoplasmic Ca\textsuperscript{2+} concentrations in the low micromolar range.

Similar to the plasma membrane H\textsuperscript{+}-ATPase and ER Ca\textsuperscript{2+}-ATPase, the plasma membrane Ca\textsuperscript{2+}-ATPase is inhibited by low concentrations of orthovanadate (12, 13, 15, 18). This would suggest involvement of a phosphoenzyme intermediate in the reaction mechanism of the enzyme and indicate that it is an E\textsubscript{1}E\textsubscript{2}-type transport ATPase (16). However, the plasma membrane Ca\textsuperscript{2+}-ATPase differs from these other transport enzymes in having a broader specificity for nucleoside phosphates compounds as substrates. While the plasma membrane H\textsuperscript{+}-ATPase and ER Ca\textsuperscript{2+}-ATPase are substrate specific for ATP (4, 11), the plasma membrane Ca\textsuperscript{2+}-ATPase can utilize GTP (12, 13, 19, 23) or ITP (13, 19) for driving \textsuperscript{45}Ca\textsuperscript{2+} uptake in isolated vesicles. The initial rate of \textsuperscript{45}Ca\textsuperscript{2+} uptake by these two alternative substrates is about 50 to 65% of the rate observed with ATP (12, 13, 19) and for GTP the properties of \textsuperscript{45}Ca\textsuperscript{2+} uptake were shown to be similar to those observed for ATP (23). The plasma membrane Ca\textsuperscript{2+}-ATPase and H\textsuperscript{+}-ATPase also differ in terms of their pH optimum for activity. In contrast to the relatively sharp pH optimum at 6.5 observed for the plasma membrane H\textsuperscript{+}-ATPase (4 and references therein), the plasma membrane Ca\textsuperscript{2+}-ATPase demonstrates a broader pH profile with an optimum between pH 7.0 and 7.5 (12, 13, 18, 23). However, this pH profile is similar to what is observed for the ER Ca\textsuperscript{2+}-ATPase (11 and references therein).

The plasma membrane Ca\textsuperscript{2+}-ATPase shows a profound sensitivity to the iodinated fluorescein derivative, erythrosin B. Although this compound has been shown to substantially inhibit (>85%) activity of the tonoplast H\textsuperscript{+}-ATPase and plasma membrane H\textsuperscript{+}-ATPase at concentrations exceeding 100 \( \mu \)M (7), the plasma membrane Ca\textsuperscript{2+}-ATPase can be fully inhibited at erythrosin B concentrations less than 1 \( \mu \)M (13, 19, 20, 23). Thus, sensitivity to this compound when used at low concentration might be useful as a marker for the plasma membrane Ca\textsuperscript{2+}-ATPase.

ROLE OF A nH\textsuperscript{+}/Ca\textsuperscript{2+}-ANTIPORT IN Ca\textsuperscript{2+} EFFLUX AT THE PLASMA MEMBRANE

The observation in several studies (12, 13, 18) that ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} transport in plasma membrane vesicles was insensitive to protonophores would suggest that Ca\textsuperscript{2+} efflux at the plasma membrane is mediated by the Ca\textsuperscript{2+}-ATPase and a nH\textsuperscript{+}/Ca\textsuperscript{2+} antiport driven by \( \Delta \mu \)H\textsuperscript{+} (established by the H\textsuperscript{+}-ATPase) is not present. This was further supported by the inability of imposed pH gradients to drive \textsuperscript{45}Ca\textsuperscript{2+} transport in plasma membrane vesicles from red beet storage tissue (12). However, an exception to these general observations has appeared recently for plasma membrane vesicles from maize leaves (14), where evidence for the presence of two parallel systems for Ca\textsuperscript{2+} efflux at the plasma membrane (Ca\textsuperscript{2+}-ATPase, nH\textsuperscript{+}/Ca\textsuperscript{2+}-antiport) was presented. In this vesicle preparation, ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} transport was partially inhibited by a protonophore (CCCP) and an imposed pH gradient could drive \textsuperscript{45}Ca\textsuperscript{2+} transport. The reason for the presence of these two Ca\textsuperscript{2+} transport systems in this one recent study is currently unknown. It should be noted that the plasma membrane Ca\textsuperscript{2+}-ATPase itself may act as a direct ATP-fueled nH\textsuperscript{+}/Ca\textsuperscript{2+}-antiport, since it has been shown that H\textsuperscript{+} flux may accompany Ca\textsuperscript{2+} movement (18). This H\textsuperscript{+} flux may represent a means of charge neutralization since the uncompensated flux of a divalent cation would represent a highly (and most likely prohibitive) electrogenic event.

CALMODULIN STIMULATION OF THE PLANT Ca\textsuperscript{2+}-ATPase

In animal cells, the plasma membrane Ca\textsuperscript{2+}-ATPase is regulated by calmodulin and it is proposed that this serves to activate the pump to remove cytoplasmic Ca\textsuperscript{2+} following signal transduction (22). Although there is evidence for the role of calmodulin in plant regulatory events (17), calmodulin effects upon the plasma membrane Ca\textsuperscript{2+}-ATPase have proven to be a controversial topic. Results have been highly variable depending on the plant species being examined, on whether Ca\textsuperscript{2+} transport or Ca\textsuperscript{2+}-ATP hydrolytic activity was being measured, and possibly even on the vesicle systems which have been used in these studies. For example, calmodulin was found to stimulate ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} transport twofold in plasma membrane vesicles from spinach leaves (15), while no effect on \textsuperscript{45}Ca\textsuperscript{2+} transport was observed in plasma membrane vesicles from C. communis (13) or in a plasma membrane-enriched (but Golgi-containing) vesicle fraction from pea (5).

Although calmodulin was shown to stimulate Ca\textsuperscript{2+}-dependent ATP hydrolytic activity in plasma membrane vesicles from maize roots (21) and radish seedlings (20), in the latter case there did not appear to be a correspondence between the level of calmodulin stimulation of ATP hydrolytic activity (up to 75%) and ATP-driven \textsuperscript{45}Ca\textsuperscript{2+} transport (7–8%). The use of calmodulin antagonists as a means to examine calmodulin interactions with the Ca\textsuperscript{2+}-ATPase has also yielded inconsistent results (cf. refs. 5, 13, 21) and should be viewed with caution since some antagonists (trifluperazine, W-7) can appear to inhibit ATP-driven Ca\textsuperscript{2+} transport in plasma membrane vesicles by enhancing Ca\textsuperscript{2+} efflux (13).

The lack of a calmodulin effect with plant membrane fractions could be related to the presence of sufficient endogenous (bound) calmodulin to mask any effect of added exogenous calmodulin (8, 23). Endogenous calmodulin can be removed from membrane fractions to some extent by repeated

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EGTA washing of the fraction (23 and references therein), although this may prove ineffective for some plant membrane preparations (8). The presence of endogenous calmodulin explained the lack of a calmodulin effect in red beet plasma membrane vesicle preparations since calmodulin stimulation of ATP (or GTP)-driven \(^{45}\text{Ca}^2+\) transport could be observed after repeated washing of the vesicle preparation with EGTA (23). However, the same approach was unsuccessful when used for plasma membrane vesicles isolated from \(C.\ \text{communis}\) (13).

An alternative explanation for the lack of a calmodulin effect upon the \(\text{Ca}^{2+}\)-ATPase could be partial proteolysis of the enzyme during the preparation of vesicle fractions. As shown for the animal cell plasma membrane \(\text{Ca}^{2+}\)-ATPase, partial proteolysis can result in irreversible stimulation of the enzyme and insensitivity to calmodulin (22 and references therein).

**STRUCTURE OF THE PLASMA MEMBRANE**

**\(\text{Ca}^{2+}\)-ATPase**

As with other \(\text{E}_{\text{E2}}\)-type transport ATPases (16), the plasma membrane \(\text{Ca}^{2+}\)-ATPase appears to consist of catalytic subunits that are large integral membrane proteins. At present, two strategies have been used to investigate the protein structure of the enzyme. Using antibodies to purified erythrocyte \(\text{Ca}^{2+}\)-ATPase, Briars et al. (3) were able to show cross-reactivity on Western blots with a 140 kDa peptide in a maize root \(\text{Ca}^{2+}\)-ATPase preparation. As this subunit molecular mass is similar to that observed for the animal cell plasma membrane \(\text{Ca}^{2+}\)-ATPase (22), it was concluded that this peptide represented the catalytic subunit of the plant \(\text{Ca}^{2+}\)-ATPase. Using \([\gamma-\text{P}]\text{ATP}\), these workers subsequently demonstrated a phosphorylated intermediate on a peptide of the same molecular mass which displayed properties consistent with being associated with the \(\text{Ca}^{2+}\)-ATPase (2). Based upon the unique ability of the plasma membrane \(\text{Ca}^{2+}\)-ATPase to use GTP as an alternative substrate, Williams et al. (23) used the alternative approach of forming a phosphoenzyme from \([\gamma-\text{32P}]\text{GTP}\) to determine a molecular mass for the \(\text{Ca}^{2+}\)-ATPase associated with red beet plasma membrane vesicles. This approach yielded a rapidly turning over phosphoenzyme with a molecular mass of about 100 kDa (on dodecyl sulfate PAGE) which also demonstrated properties consistent with its association with plasma membrane \(\text{Ca}^{2+}\)-ATPase. While the reason for this difference in molecular mass between the two studies is uncertain, it is possible that the lower molecular mass of the red beet enzyme could result from partial proteolysis of the enzyme preparation (23 and references therein). Studies with the erythrocyte \(\text{Ca}^{2+}\)-ATPase (22, 23 and references therein) have shown that limited proteolytic digestion of the enzyme can result in fragments with molecular masses in the 71 to 90 kDa range which can retain the capacity for ATPase activity, \(\text{Ca}^{2+}\) transport, and calmodulin stimulation (depending on the site of proteolysis).

Preliminary studies involving radiation inactivation analysis have also suggested that the plasma membrane \(\text{Ca}^{2+}\)-ATPase may exist as a multimeric enzyme in the native membrane. Using this approach, Rasi-Caldogno et al. (20) found a target molecular size of about 270,000 for both \(\text{Ca}^{2+}\)-dependent ATPase activity and ATP-dependent \(^{45}\text{Ca}^2+\) transport in radish seedling plasma membrane vesicles. As this target molecular size generally estimates the native molecular mass of an enzyme, this would suggest that the native enzyme may exist as dimeric or trimeric collection of catalytic subunits depending on whether the catalytic subunit has a molecular mass of 100 or 140 kDa.

**SUMMARY AND FUTURE PERSPECTIVE**

Although there was initial uncertainty regarding the presence of a plasma membrane \(\text{Ca}^{2+}\)-ATPase in higher plant cells, current work with isolated membrane fractions argues for the presence of such an enzyme which would be involved in mediating active \(\text{Ca}^{2+}\) efflux at the cell surface. Characterization of \(\text{Ca}^{2+}\) transport activity associated with this protein has shown unique characteristics which allow it to be distinguished from other transport ATPases associated with higher plant cells. However, a substantial amount of work needs to be done to further understand the structure and regulation of this transport enzyme. The possible interaction of the \(\text{Ca}^{2+}\)-ATPase with calmodulin needs to be clarified to determine whether, as in animal cells, this regulatory protein serves to modulate \(\text{Ca}^{2+}\)-ATPase activity in accordance with cytoplasmic \(\text{Ca}^{2+}\) concentration. The application of molecular approaches to the plasma membrane \(\text{Ca}^{2+}\)-ATPase may greatly facilitate efforts to elucidate the structural details of this protein and allow its comparison to the more well-characterized transport enzymes.

**LITERATURE CITED**

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